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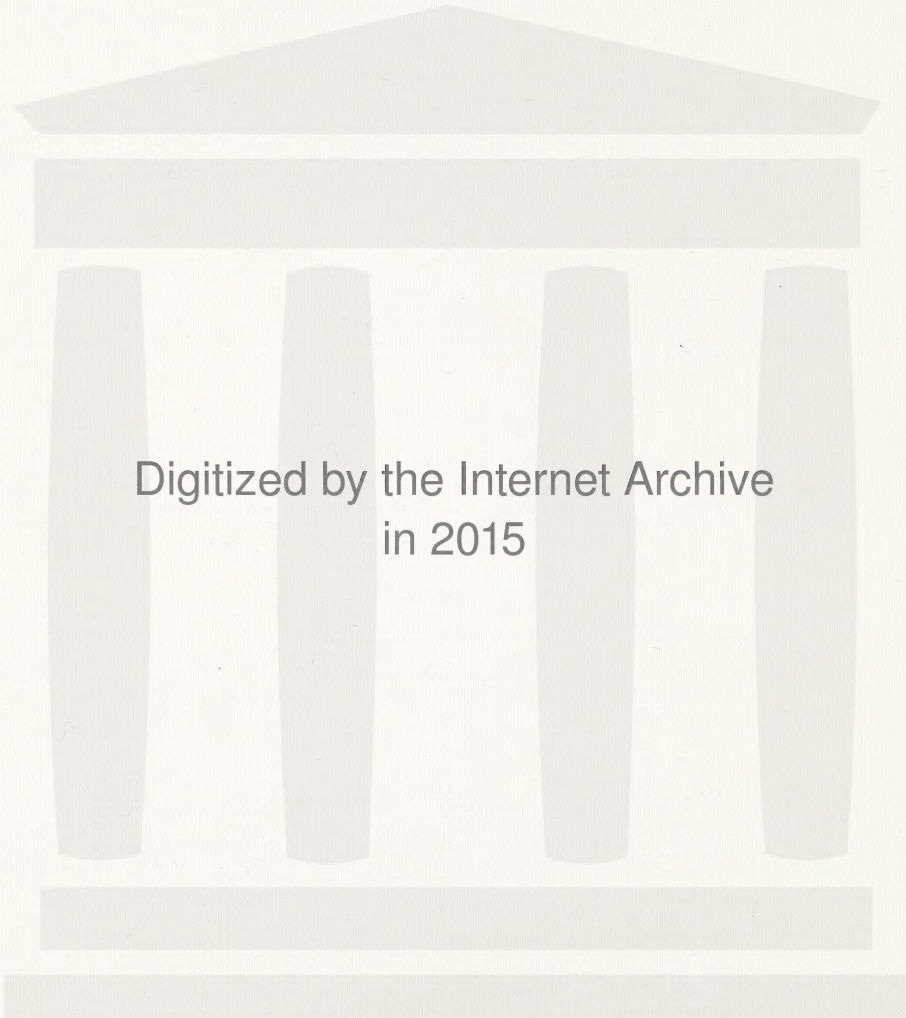

# DISCUSSION OF ALTERNATIVES TO THE DRAIZE EYE IRRITANCY TEST

by

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# PREFACE

This document was prepared originally for the Animal Care and Use Committee of the Alberta Environmental Centre to serve as a basis for discussion of some alternatives to the Draize Test for ocular irritancy. The Committee wished to have information about the search for more humane methods of assessing eye irritation. Subsequent revisions have included additional contemporary information.

This document does not represent the official policies, views or opinions of either the Animal Care and Use Committee or the Alberta Environmental Centre.

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# 1 INTRODUCTION

The identification of potential ocular hazards of commercial products (e.g., foodstuffs, cosmetics, pesticides, household and industrial chemicals) is a requirement for regulatory approval. The Draize eye irritancy test (Draize *et al.*, 1944; Friedenwald *et al.*, 1944) is an *in vivo* procedure required by many regulatory agencies for the testing of such commercial products. The test was developed in the early 1940's and has been used extensively since its inception. In recent years, it has come under considerable criticism from both animal welfare groups and the scientific community. Consequently, efforts have been made to develop alternatives for the Draize test. Recently, several pharmaceutical and cosmetic companies have started to apply alternative tests to replace some of the ocular irritancy testing, for example, Noxell Corp., Mary Kay Cosmetics, and Avon Products (Anon., 1989a, 1989b, 1989c; Holden, 1989; Anon., 1990).

In spite of these intensive efforts to develop and adopt alternative tests, none have been generally accepted (Gilman, 1991). Until viable alternatives for the Draize test are established, the test will continue to be used with modifications to reduce the number of animals used, improve scoring, and/or reduce stress gaining further acceptance (Anon., 1986a; Taniguchi *et al.*, 1988; Li and Zhan, 1990; Morgan *et al.*, 1990; Gilman, 1991).

This report provides discussion on the development of alternatives and also modifications for the Draize ocular irritancy test. It provides information for assessing the validity and use of these alternatives to the Draize test. Because of the complexity of related research, this report has only highlighted those alternatives which show potential as replacements for the Draize test. The evaluation and use of alternative ocular irritation tests at the Alberta Environmental Centre is part of an ongoing review by the Animal Care and Use Committee for animal-based research procedures.

# 2 DRAIZE TEST

## 2.1 Description

The Draize test generally involves the application of an aliquot of the test article (usual dosage of 0.1 mL or 0.1 g) underneath the lower eyelid of one eye in albino rabbits (Draize *et al.*, 1944; Friedenwald *et al.*, 1944). The other eye is left untreated to serve as a control. The upper and lower lids are held together to allow the test substance to distribute over the ocular

surface. Six rabbits are used for each test. The rabbits are held in restraining cages during the application of the test substances to immobilize them and prevent undue injury to either researchers or rabbits.

Following application, the eyes are inspected at selected intervals (eg. 1, 12, 24, 48 h). Damage to the eyes (eg. inflammation of the conjunctiva and iris, clouding of the cornea) is evaluated according to published scales (see Appendices A and B).

The Draize test is a widely used method for the assessment of ocular irritancy potential of several groups of products used by people. There are four product categories for which eye irritation assessment studies are required (Swanson, 1991):

1. pharmaceuticals (eye therapeutics),
2. cosmetics and toiletries (make-up, shampoo, soap, etc.),
3. consumer products (household detergents and chemicals),
4. industrial chemicals (hazardous chemicals handled by workers).

Frazier *et al.* (1987b) have identified the following rationale for the use of the Draize test:

1. provides a whole animal and organ evaluation,
2. complete products or specific chemicals can be tested in concentrated or dilute form,
3. assessment of the recovery and healing process,
4. several regulatory Acts [e.g., Federal Hazardous Substances Act (FHSA, 1974); Environmental Protection Agency Toxic Substances Control Act (EPA/TSCA, 1985)] require information on eye-irritation,
5. yields quantitative and qualitative information under the Draize scoring system,
6. the test can be easily modified,
7. a rabbit is easy to handle,
8. the ocular surface area of the albino rabbit eye is large and it is easy to interpret degree of inflammatory response,
9. the Draize test is a conservative measure of eye-irritation that errs in favour of people.

Since its first description, the procedure has remained relatively unchanged. There have been several attempts to refine the procedure, however. For example, a high level of accuracy can still be obtained when reducing the number of rabbits from six to three per test (De Sousa *et al.*, 1984; Talsma *et al.*, 1988; Bruner *et al.*, 1992). Other modifications have centred on improving scoring methods and reducing stress (Gilman, 1991).

## 2.2 Criticisms of the Draize Test

Aside from the ethical considerations of inflicting pain on conscious animals, scientific criticism of the Draize test falls into three categories (Stephens, 1986):

a. Reproducibility. In the evaluation of 24 laboratories, variable results were obtained both between and within testing laboratories (Weil and Scala, 1971). Several laboratories consistently reported either greater irritation or less irritation for the same test substances than other laboratories. Although variability in the performance of the test may have been responsible for these differences, the primary reason was in the evaluation of damage in the eye.

b. Subjectivity. The score is subjective and to a large extent is unable to precisely measure the extent of ocular damage. The scoring system uses words to describe injuries which can be interpreted differently by each laboratory. No objective empirical measurements are taken to assess the damage associated with the instillation of test chemicals. The test is only able to crudely classify whether substances will be irritants or not. Statistical analyses of the results may be questionable.

c. Validity. The test results may not be applicable to humans. Structurally, there are considerable differences between the human and rabbit eye. For example, the rabbit eye has a thinner cornea (0.37 mm compared to 0.51 mm in humans), larger corneal coverage (25% of total global area in rabbits compared to 7% in man), produces fewer tears, and possesses a nictitating membrane (Robinson, 1984; Frazier *et al.*, 1987a). These differences make extrapolation of Draize results to humans difficult.

Additional objections to this test also focus on the ethical unacceptability of the test itself (Frazier *et al.*, 1987b; Schlatter and Reinhardt, 1985; Sharpe, 1985; Swanston, 1985; Scaife, 1985; Anon., 1986b).

These criticisms indicate that there is a need to develop alternatives to the Draize test. Two technical approaches have been the focus of researchers.

- Refinements of the Draize test
- Development of new *in vitro* tests

## 3 REFINEMENTS OF DRAIZE TEST

Listed below are proposed modifications for the Draize test. These changes may reduce the suffering of the animals and/or improve the quantitative evaluation of the test (Stephens, 1986; Frazier *et al.*, 1987a):

- use of alternative species
- use of anaesthetics or antihistamines
- use of smaller doses and fewer animals
- use of prescreen tests
- use of objective empirical measurements

### 3.1 Alternative Species

Dogs, monkeys, or mice have been suggested as test subjects instead of rabbits (Swanston 1985; Frazier *et al.*, 1987a). Associated problems in costs, handling, and lack of adequate biological databases may preclude their use. The use of alternative species in testing fails to address the problems of pain and stress induced by the test procedure.

### 3.2 Anaesthetics and Antihistamines

Anaesthetics, either topical or general, have been proposed as a means of reducing the pain associated with the instillation of test chemicals in the eye (Maurice, 1985; Ulsamer *et al.*, 1977). The use of any anaesthetic, however, must not interfere with the Draize test (e.g., decreasing the tear response or increasing the permeability of the test substance). Proparacaine (0.1%) was found to be a suitable anaesthetic because it had no interfering effects on the test (Maurice, 1985).

Swanston (1983) has suggested the use of antihistamines instead of anaesthetics to alleviate pain. However, insufficient information exists to warrant their use or to demonstrate their effectiveness. In fact, the use of antihistamines may be counterproductive due to their anti-inflammatory effect in tissues scored by the Draize test.

### 3.3 Smaller Doses

The dose commonly used in the Draize test is 0.1 mL or 0.1 g. However, this dose of material can be above the fluid holding capacity of the eye. Consequently, the excess dose may be expelled and not absorbed or retained in the eye. It has been suggested that the use of low doses (0.01 mL or 0.01 g) might be more realistic, give better results and cause less eye damage (Griffith *et al.*, 1980; Stephens 1986; Williams, 1983, 1984). Factors that may be important in the design of eye irritancy testing are the concentration of the test substance and the duration of contact before washing (Murphy *et al.*, 1982). Low dose responses can be less severe, less stressful, shorter in duration, and more sensitive in discriminating between similar substances (Griffith and Freeberg, 1987).

### 3.4 Reduction in Number of Animals

Because of the requirements from regulatory agencies, the Draize test routinely requires six rabbits for each test (DeSousa *et al.*, 1984). However, reducing the number to three rabbits has shown no significant difference with the Draize values from the six rabbit protocol (DeSousa *et al.*, 1984; Hatoum *et al.*, 1987; Talsma *et al.*, 1988; Bruner *et al.*, 1992). Other reduction schemes may be equally valid, but require review by statisticians to ensure that they produce statistically valid results and overcome the inherent subjective evaluation schemes.

### 3.5 Prescreen Tests

Prescreen tests based on the physicochemical properties of the test substance (eg. pH, redox potential), primary dermal irritation tests, and staggered eye testing may reduce the need for the complete Draize test. European Chemicals Industry Ecology and Toxicology Centre (ECETOC, 1988) has recommended that a stepwise test strategy, utilizing a variety of prescreening evaluations, be implemented for assessing ocular irritation (Figure 1).

Talsma *et al.* (1988) proposed that the complete Draize test may not be necessary if several factors are initially considered. First, the structure and reactivity of the test chemical are examined and compared with the results for similar chemicals. Second, the pH of the chemical solution/suspension is measured and chemicals with a pH of less than 2.0 or greater than 11.5 are assumed to be eye irritants. Lastly, if dermal applications caused irritation, then modified eye irritation studies can be started. These tests may involve staggered testing where the test substances are applied at 2 hour intervals. For example, the first animal can be tested with a dilute sample of the agent. After 2 hours, the animal is inspected for ocular injury. If serious injury is present, the testing is discontinued or a greater dilution is used. In this manner, the number of animals used for testing can be reduced significantly. Similar reduction schemes can be devised as prescreen testing.

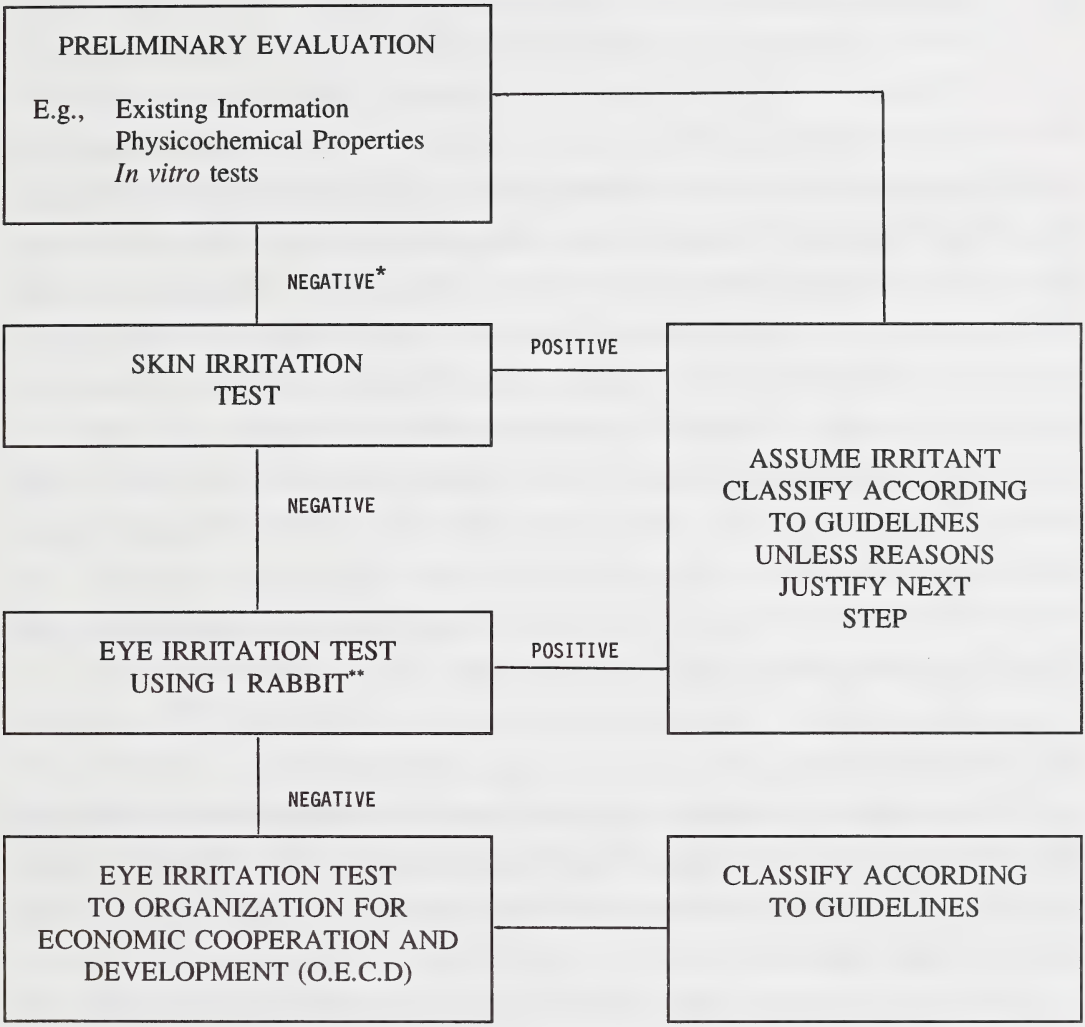
However, caution should be exercised in basing the requirement for further ocular irritation testing completely on the prescreen testing. Hydrochloric acid (0.3%, pH 1.28) and 5% citric acid (pH 2.1) were found to induce no corneal opacity. On the other hand, 5% phenol (pH 7.7) and 5% acetic acid (pH 2.7) were both capable of producing corneal opacity. Similarly, 0.3% (pH 12.8) and 0.1% (pH 12.3) sodium hydroxide did not produce opacity while 1% (pH 13.1) did (Murphy *et al.*, 1982). Consequently, the European Community has ruled out the use of pH as predictor of ocular irritancy (personal communication).

Dermal irritation test results may also be a poor prescreening procedure of ocular irritancy. In the testing of 60 severe dermal irritants, only 39 were found to be severe eye irritants while 15 were mild or non-irritants (Williams, 1983, 1984). It may be possible that the dermal test results were over-estimated by the current methods.

### 3.6 Objective Empirical Measurements

Several methods have been used to overcome the subjective nature of the Draize test and provide objective empirical information.

- Exfoliative cytology
- Fluorescent dyes
- Corneal thickness
- Fluid biochemistry




* The negative and positive criteria are defined by either (a) the experimentalist or (b) international guidelines.

** At the *in vivo* level the use of diluted material, a reduced volume or anaesthesia may be appropriate as part of the stepwise policy for assessment of ocular irritation.

FIGURE 1 AN EXAMPLE OF A STEPWISE STRATEGY FOR ASSESSING OCULAR IRRITATION (ECETOC, 1988)

### 3.6.1 Exfoliative Cytology

A group at Rockefeller University has proposed the collection and quantification of exfoliated inflammatory cells from the treated eyes (Walberg, 1983; Stark *et al.*, 1983, 1985). While inspecting the eyes in the Draize test, the corneas of the experimental and control eyes were also gently rinsed with 0.25 mL of warm distilled water. The wash was collected and the total number and type of cells were determined. The cells were concentrated, fixed, and stained for light microscopy. This procedure was objective, able to predict the irritancy of the dilute chemicals and well-tolerated by the animals (Shopsis *et al.*, 1985). This procedure was modified for use in rats and was found to be effective in assessing the eye irritation resulting from exposure to two sublethal concentrations of hydrogen sulphide gas (Lefebvre *et al.*, 1991).

However, certain cautions should be exercised in the evaluation of the exfoliative cytology procedure. Shopsis *et al.* (1985) noted that one of the test chemicals (10% sodium dodecyl sulphate) produced significant swelling of the conjunctivae and nictitating membrane. This swelling made it difficult to rinse the cornea. Although large quantities of cells were recovered, they tended to clump and quantification was impossible.

### 3.6.2 Fluorescent Dyes

Corneal injuries in the Draize test can be detected by adding a drop of 2% fluorescein ophthalmic solution into each rabbit eye after 72 h post-exposure to the test chemical (Hickey *et al.*, 1973). The dye is rinsed off with saline and the cornea is inspected under a ultraviolet light for retention of the dye.

In a relatively similar procedure, Etter and Wildhaber (1985) were able to quantify the fluorescence emitted by the diffused fluorescein. Twenty minutes after treatment with the test chemical, 4 µL 0.1% sodium fluorescein was applied to the eye of the anaesthetized mouse and rinsed off after 2 minutes with physiological solution (37°C). Excitation fluorescence at 400 nm was measured 27 minutes later using an episcopic microscope ($\lambda$=546 nm max) and a photomultiplier. This procedure measures the changes in corneal permeability to fluorescein induced by ocular injury.

Modifications of this procedure include using sulforhodamine B as the fluorescent dye and testing on recently euthanized mice (Maurice and Singh, 1986; Brooks and Maurice, 1987).

However, Maurice and co-workers suggest that this test be used only as a rapid screen for acute corneal injury rather than long-term delayed responses.

### 3.6.3 Corneal Thickness

Several authors have suggested that corneal thickness may be an adequate measure of ocular irritancy, since a majority of the Draize score is dependent on corneal damage (Burton, 1972; Kennah *et al.*, 1989b). At the time of Draize scoring, the rabbit would be anaesthetized and the eye is placed in front of the biomicroscope. The slit-lamp biomicroscope presents a cross-sectional view of the cornea and the image is split in half by a pachymeter. By aligning the 2 half images, the thickness of the cornea can be determined. Morgan *et al.* (1987) found that the highest correlation between corneal thickness and corneal opacity was observed on day 3 post-exposure to 7 different test chemicals.

### 3.6.4 Fluid Biochemistry

Another possible measure is the biochemical analysis of conjunctival fluids (e.g., tears and aqueous humour) for inflammatory mediators such as histamines, serotonin and leukotrienes. These chemicals in conjunctival fluids would provide an objective measurement of the inflammatory response following ocular insult (Benassi *et al.*, 1987). Following the Draize test, 50 µL of a balanced salt solution containing an internal standard was instilled into the eye. After 10 seconds, 20 µL of this lavage fluid was collected and half was directly injected into the high pressure liquid chromatograph (HPLC) to measure the dilution factor of the internal standard. The remaining half was derivatized with fluorescamine before HPLC analysis to measure histamine and serotonin levels.

## 4 *IN VITRO* ALTERNATIVES

There has been considerable research conducted to find alternatives to the Draize test. The criteria for the development of the alternative tests (Bruner *et al.*, 1991) are:

- the endpoint of the *in vitro* assay must correlate in a predictable manner with the *in vivo* biological response being monitored;
- the *in vitro* assays should have a biological basis linking them to the processes occurring in ocular injury and;

- the *in vitro* tests must be technically sound and relatively easy to conduct.

The alternative tests can be broken down into five major groupings:

- Chorioallantoic membrane
- Non-target organ assessment
- Enucleated eyes
- Cytotoxicity assays
- EYTEX™ Method

## 4.1 Chorioallantoic Membrane

### 4.1.1 Method

The Chorioallantoic Membrane (CAM) test has demonstrated promising potential as a possible replacement for the Draize test (Leighton *et al.*, 1983). In the developing chick embryo, the CAM acts as the respiratory organ and exists for the initial 2 weeks of the incubation period.

The procedure, with all of its variations, is well-documented in recent literature (Leighton *et al.*, 1983, 1985; Luepke, 1983, 1985; Luepke and Kemper 1986). In general, fertile hen's eggs are incubated at 37°C. At day 3, 1.5 to 2.0 mL of albumin is removed from the pointed end of the egg using a syringe. An opening is made on the lateral surface of the shell and closed with transparent tape. On day 14, the tape is removed and a Teflon ring (10 mm i.d.) is placed on the CAM. The test sample is placed in the ring, tape replaced and the egg returned to the incubator. The CAM is examined on day 17 (Leighton *et al.*, 1983, 1985) for inflammation, hemorrhages or coagulation.

### 4.1.2 Critique

The CAM has several advantages for use as a test subject. Primarily, it possesses no nerve fibers for the sensation of pain. It is economical since eggs are relatively inexpensive and require little maintenance.

Test results provided a measure of the inflammatory response and the reparative process following treatment with the test substance. The CAM test was found to correlate well with the

Draize test for the same chemicals (Leighton *et al.*, 1985; Luepke 1985; Luepke and Kemper 1986).

Its suitability as an alternative procedure for the Draize test, however, is disputed. Price *et al.*, (1986) found that the CAM test does not possess the necessary predictive ability for irritation potential. In their study, if the criteria for evaluation was the presence or absence of a response, then the CAM test correlated well with the Draize test. However, there was no correlation between the degree of inflammation, hemorrhaging, or coagulation and the degree of ocular damage found in the Draize test. The CAM test consequently should not be used as a screen to assess the hazard potential of the test substances (Price *et al.*, 1986).

Lawrence *et al.* (1986) reported that glycerine, polyethylene glycol and Tween 80 produced positive results at more than one concentration for the CAM test and yet, these chemicals are generally classified as non-irritants by the Draize test. The CAM test is not a suitable alternative to the Draize test because the causes of the inflammation are different between the eye and the chick embryo (Lawrence *et al.*, 1990b).

In the testing of 47 different surfactants, Kong *et al.* (1987) found that the CAM test was unable to discriminate between the positive and negative known irritants. In a test of 12 anionic surfactants, the CAM test was unable to predict eye irritation potential upon comparison with a modified Draize test using guinea pigs (Reinhardt *et al.*, 1987). During validation tests, the CAM procedure was found to be too sensitive for undiluted samples (r=0.348), but better correlations with the Draize results (r=0.670) were obtained when using diluted tests chemicals (Blein *et al.*, 1991). The utility of the CAM procedure as an alternate model is also questionable due to its high false positive rates, the high death rate of the chicks developing within the treated egg, and the lack of a classic inflammatory response (Bruner *et al.*, 1991). Attempts to improve the CAM procedure so that the inflammatory responses are similar to those in the eye have failed because any pretreatment of the CAM may mask the effects of the irritant chemical (Friend *et al.*, 1990).

Another source of error in the CAM test may be the genetic heterogeneity of the chicken. Unlike rabbits which may be inbred for generations, the control of chicken inbreeding may not be as rigid (Leighton *et al.*, 1985). In addition, differential thickness of the CAM within and between eggs may also be responsible for the inaccuracies of the CAM test with thicker sections allowing a substrate for the formation of granulation tissue (Leighton *et al.*, 1985). Kalweit *et*

*al.* (1990) observed that the results of the CAM test were dependent upon the level of experience in the investigators.

## 4.2 Non-target Organ Assessment

### 4.2.1 Method

An associated problem in the development of a Draize test alternative has been the presence of a penetration barrier in the eye (Robinson, 1984). Most cytotoxic alternatives eliminate the barrier factor and hence, may not be representative of an eye exposed to an irritant. To incorporate this aspect, Muir *et al.* (1983) developed an assay using sections of rabbit ileum as an alternative to the Draize test. The ileum of the rabbit has a penetration barrier, and this feature allows it to be a good *in vitro* model to determine barrier related problems for toxicity assessment.

In this test, segments of rabbit ileum are placed under a resting tension of 1.0 g in aerated organ baths of 37°C Ringer's solution. Using isotonic transducers and an oscilloscope, spontaneous contractions of the segments are recorded for 25 minutes. Test chemicals were then added at 10 minute intervals and changes in spontaneous contraction rates were correlated with the Draize values.

### 4.2.2 Critique

A major advantage of the rabbit ileum procedure is that one rabbit can provide as many as 16 segments of ileum for testing (Robinson, 1984). This will reduce the number of animals to be sacrificed for the testing procedure. However, the test makes certain assumptions regarding the similarity of rabbit ileum and corneas which may limit the interpretation of results (Frazier *et al.*, 1987a). It assumes that the penetration barrier and chemical exclusion nature of both the ileum and cornea are practically similar.

## 4.3 Enucleated Eyes

### 4.3.1 Method

Eyes from euthanized rabbits were immediately dissected out and examined for damage before use. Eyes that were significantly swollen, stained with fluorescein (after vital staining with fluorescein), or damaged otherwise were rejected. The enucleated eyes were clamped in temperature regulated chambers with saline dripping over the surface at regular intervals (Burton *et al.*, 1981; Price and Andrews, 1985). The eyes were allowed to equilibrate in the chambers for 45-75 minutes. Solutions of chemicals (0.1 mL) were then dripped onto the eye and left for approximately 10 seconds. The treated eyes were then carefully rinsed with saline and permeability changes, corneal opacity, and corneal swelling were measured (Koeter and Prinsen, 1985). The corneal thickness of enucleated rabbit eyes was measured following treatment with various chemicals and found to correlate well with the Draize test results (Price and Andrews, 1985).

Benassi *et al.* (1987) measured the concentration of inflammatory mediators (e.g., histamine, leukotrienes and serotonin) in enucleated bovine eye cups treated with various chemicals. Eyes were obtained from slaughterhouse animals immediately after death. The muscles, anterior portion of the eyes (cornea, lens, ciliary bodies), vitreous humour and retina were removed. The remaining "cup" is immersed in buffer solutions and allowed to equilibrate for 15 minutes. The test chemical is added to the medium and serial sampling of the media is done for 90 minutes. Histamine and leukotriene levels are assayed by fluorescamine derivatization and HPLC (Frazier *et al.*, 1987a). The amounts of inflammatory mediators released by ocular tissue appeared to be good indicators of ocular irritancy.

### 4.3.2 Critique

One of the major criticisms of the Draize test has been the infliction of pain to the conscious animal. Although refinements in the Draize test may reduce the discomfort or pain to some degree, elimination of the pain would require the replacement of the living animal with a nonliving model. The enucleated eye system provides such a complementary model in which the pain component is eliminated because the animals are humanely euthanized before their eyes are used. In addition, rabbit eyes can be substituted with eyes from other species. For example,

bovine and pig eyes can be easily obtained from local slaughterhouses and hence, forego the expenditures of animal housing. Many of the objective measurement techniques mentioned in Section 3.6 can be applied on enucleated eyes.

The results of the enucleated eye procedure correlated well with the Draize test (Koeter and Prinsen, 1985; Weterings and Van Erp, 1987). The advantage of this procedure over the Draize test is its ability to check severe irritants without concerns raised about pain or stress. The procedure would allow testing of a wide variety of test agents and dosages as is without dilution or other modifications (York, 1983). Other advantages of this test are the reduced animal care costs as compared to the Draize test and the ability to complete/evaluate the test within one day (Koeter and Prinsen, 1987).

Criticisms of the use of enucleated eyes have focused on the inability of the procedure to measure long-term and reparative effects. As well, animal usage cannot be reduced since one animal can only provide two eyes for testing as is the case in the Draize test (Muir *et al.*, 1983). Other disadvantages of the test are the requirement for non-standard equipment, lack of standardized scoring criteria and the inability to automate the system (Koeter and Prinsen, 1987).

In addition, more work is required to develop objective criteria. At present, the database on the levels of inflammatory mediators, histamine and leukotriene, released in the presence of ocular irritants is small (Frazier *et al.*, 1987a). When using eyes from a slaughterhouse, concerns may be raised regarding the maintenance of sample quality, infection of the enucleated eyes, availability, and transportation problems.

## 4.4 Cytotoxicity Assays

Numerous *in vitro* cytotoxicity assays have been proposed as replacement tests for the Draize test. Cell cultures used for this purpose have included ocular, non-ocular, and even protozoan types (Silverman, 1983; Silverman and Pennisi, 1985). Some of the proposed test systems to be discussed are:

- Growth inhibition of mouse fibroblast cells
- Cellular uptake
- Biochemical assays

- Ocular cell cultures
- Corneal epithelial wound repair
- Agarose diffusion method

4.4.1 Growth Inhibition of Mouse Fibroblast Cells

Growth inhibition of mouse fibroblast Balb/c 3T3 cells was examined as a potential alternative by Shopsis *et al.* (1985). Balb/c 3T3 cells were seeded into 96-well plates for 24 h. The medium was then replaced with test media containing the test chemicals in various concentrations. After 24 h incubation, the cells were scored for morphological alterations. The Draize test results were found to correlate with the ability of chemicals to induce morphological changes in the established cell line.

In addition, a known concentration of Balb/c 3T3 cells were plated in a petri dish for 24 h. The media was then replaced with test chemicals in various concentrations for another 24 h. The cells were subsequently washed and allowed to grow in normal medium for 7 days. Colonies of Balb/c 3T3 cells were then counted. The ability of Balb/c 3T3 cells to form colonies in the presence of test substances correlated well with the Draize test.

4.4.2 Cellular Uptake

A modification of the above cell growth assay was the measurement of uptake and release of certain markers of cell viability, e.g., [$^{3}$H]-uridine (Shopsis and Sathe, 1984; Shopsis *et al.*, 1985; Stark *et al.*, 1983, 1985), neutral red (Hockley and Baxter, 1986), $^{51}$Cr (Shadduck and Everitt, 1985), crystal violet (Itagaki *et al.*, 1991), fluorescein diacetate and ethidium bromide (Shaw *et al.*, 1991; Scaife, 1985) by various cell lines (e.g., Balb/c 3T3, HEp2, MDCK, and HeLa). Briefly, a known concentration of cells were plated for 24-48 h. The growth media was then replaced with media containing various concentrations of the test agent. After a previously established incubation period, the cells were washed with buffer and incubated with media containing the indicator chemical. The cells were then washed and lysed. The lysate was analyzed for the indicator chemical. The permeability and retention of these chemicals is dependent on the integrity of the cellular membranes and the metabolic activity of these cells. Insults of cytotoxic chemicals may affect the ability of the cells to take up and retain the indicator chemicals.

4.4.3 Biochemical Assays

Cytotoxicity studies have also investigated the effects of test chemicals on biochemical or metabolic endpoints in the same cell lines in attempts to correlate with the Draize test results. The premise for the biochemical assay is that treatment with irritant chemicals can change the metabolic status of the cell and related changes can be detected. For example, one such endpoint for cytotoxicity is alteration in ATP concentration. It was demonstrated that this assay is an extremely sensitive test of cellular viability and may prove to be a reliable alternative to the Draize test (Kemp *et al.*, 1985). Mouse fibroblast cell cultures were established and exposed to cosmetic products for 4 hours. The cultures were then analyzed for ATP levels using the firefly luciferase-luciferin assay (Kemp *et al.*, 1985). Other biochemical endpoints proposed as Draize test alternatives are cellular protein levels in Balb/c 3T3 cells (Shopsis and Eng, 1985); reduction of MTT [3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide] by mitochondria in human keratinocytes and fibroblasts (Cornelis *et al.*, 1992; Sina *et al.*, 1992); and leukocytic chemotactic factors in bovine and rabbit corneal cultures (Elgebaly *et al.*, 1987). The advantages of such assays are their sensitivity, speed and automation capabilities.

4.4.4 Ocular Cell Cultures

Similar cytotoxicity assays have also been developed for ocular cell cultures. Colony formation capacities of rabbit corneal cells (SIRC) were demonstrated to have good correlations in the testing of surfactants when compared with *in vivo* studies (North-Root *et al.*, 1982, 1985).

Human corneal endothelial cells have been cultured and tested as a possibility as a Draize test alternative (Douglas and Spilman, 1983). These cultured cells were found to be susceptible to various test substances. The suitability of human corneal cells as a predictor of ocular irritation potential requires further study.

4.4.5 Corneal Epithelial Wound Repair

The corneal epithelium acts as a permeability barrier in the eye and, when these cells are damaged, the surrounding epithelial cells will migrate over the wound to reestablish the barrier. An alternative test using rabbit corneal epithelial cells has been developed which mimics the corneal response to ocular injury (Jumblatt *et al.*, 1987; Simmons *et al.*, 1987).

The assay procedure entails the intentional physical wounding of cultures of stratified layers of these epithelial cells and observing the repair process when these "wounded" cell cultures are exposed to the test substances. The wound is measured by planimetry and the cells are fixed and stained for light microscopy. Results indicate that the procedure may be comparable to the Draize scores of corneal damage. Therefore, the procedure may be a viable alternative to the corneal component of whole animal testing.

However, it will not be suitable for the testing of substances on nonepithelial sites, eg. stroma, mast cells (Simmons *et al.*, 1987). Attempts to culture human epithelial cells have been unsuccessful at present (Jumblatt and Neufeld, 1985).

### 4.4.6 Agarose Diffusion Method

A major limitation of the cytotoxicity tests in the past was their inability to be useful for testing water-in-oil emulsions, water or hydrocarbon-based suspensions, gels and waxes. As well, cytotoxicity tests lack a penetration barrier which may play a vital role in the irritation potential of test agents. In order to overcome these problems, a test was developed with monolayer cultures of mouse fibroblast cells (ATCC #CCLI, NCTC clone 929, clone of strain L) overlaid with 1% agarose (O'Brien *et al.*, 1990; Jackson *et al.*, 1988; Wallin *et al.*, 1987). The agarose layer serves as a penetration barrier to the test substances and will allow non-aqueous samples to be tested for this test. Filter discs containing the agent were placed on the agarose layer and examined 24 h later for zones of cell lysis around the discs. The irritants diffuse through the agarose layer and comes in contact with the cell layer and elicit its cytotoxic effect. A toxic reaction was reported if evidence of cell death and/or degeneration was noted directly beneath the area of the test sample and possibly beyond the test sample as well. In addition, the zone of lysis was measured.

### 4.4.7 Critique

The development of cytotoxicity assays as substitutes for the Draize test has been promising. The assays appear sufficiently robust in that similar responses have been demonstrated in tests using different cell lines. Borenfreund and Shopsis (1985) showed that the highest tolerated dose (HTD) ranking of various tested chemicals was the same in 5 different cell

lines, ie. mouse Balb/c 3T3, hamster CH v79, rabbit cornea, human $HepG_2$, and mouse RAW 246.7.

In spite of the advances made in cytotoxicity assays, its use as a general or universal replacement test for the Draize procedure is still disputed (Sina *et al.*, 1992). For a number of test chemicals, Kennah *et al.* (1989a) found a poor correlation between ocular irritancy and growth inhibitions using the BALB/c 3T3 cell line. Differences in correlation may be due to failing to run an *in vivo* Draize test simultaneously with the cytotoxicity tests (Kennah *et al.*, 1989a), and relying on previously published work. Selling and Ekwall (1985) found that although morphological changes in HeLa cell cultures correlated well with ocular irritancy, two extreme irritants, allyl alcohol and 1-heptanol were not identified as such by the assay. The discrepancy may be due to the lower solubility of the two alcohols. Cytotoxicity was found not to be reliable enough to distinguish between closely related detergent-based substances (Flower, 1987). Another criticism is that, "*in vitro* cytotoxicity can be accounted for based upon physical mechanisms solely ... *in vivo* eye irritancy appears to be elicited by both chemical and physical mechanisms" (Kennah *et al.*, 1989a).

Tests based on cytotoxicity were also unable to assess the role of other factors that *in vivo* may be responsible for irritation. Factors such as the physical removal of substances from the eye, presence of penetration barriers, enzymatic reactions with test chemicals, tissue adsorption characteristics, and inflammatory mediation need to be considered (Flower, 1987; Scaife, 1983). Simple cytotoxicity tests were only able to mimic the *in vivo* response when the major difference is averted, that is, the penetration barrier was removed (Scaife, 1983).

A good correlation was found between the Agarose Diffusion Method (ADM) and the Draize irritancy test - 81% for oil-in-water emulsions, water and petroleum distillate-based suspensions, solutions, waxes, and hydroalcoholic solutions (Wallin *et al.*, 1987) and 100% for water-in-oil emulsions, water or hydrocarbon-based suspensions, gels and physical mixtures of powders or waxes (Jackson *et al.*, 1988). The mouse fibroblast cell line used in this test can be easily cultured and it requires no specialized equipment. The ADM could be run at a lower cost ($50 - $100) than the Draize test ($500 - $700). Results from ADM can be obtained within 24 h while the Draize test may require up to 21 days to complete the test (Wallin *et al.*, 1987). The procedure could even be modified using the neutral red release or the reduction of thiazolyl blue

as endpoints (O'Brien *et al.*, 1990). This test has been implemented by Noxell Corporation as a preclinical screen for product safety tests of cosmetic and skin care products (Gribetz, 1989).

## 4.5 EYTEX™ Assay

### 4.5.1 Method

A commercial procedure that shows considerable promise as an alternative is the EYTEX™ method (Lawrence *et al.*, 1990a). This test has been accepted by Avon as a replacement for the traditional Draize test (Anon., 1989b). The test does not require the use of animals or tissue cultures, but is based on the reaction of a proprietary mixture of protein aggregates with the irritant to produce an opacity similar to the exposed cornea (Gordon *et al.*, 1990; Frazier *et al.*, 1987a). The opacity can then be measured and compared with other irritants or control substances. The protein aggregate consists of globulin, albumin, carbohydrates, lipids, mucopolysaccharides, sodium acetate, sodium borate, and EDTA (Soto and Gordon, 1990).

### 4.5.2 Critique

The advantage of this system is a standardized and objective procedure which is inexpensive, easy to learn, cost effective, and quick to run (Soto and Gordon, 1990). The EYTEX™ procedure has been found to strongly correlate with the Draize test (greater than 90% predictive ability). In addition, it shows reproducibility between and within laboratories; provides empirical measurements which can be utilized for comparative purposes; and a comparable chemical database exists (Gordon *et al.*, 1990; Soto and Gordon, 1990).

Bruner *et al.* (1991), however, found very little correlation between the *in vivo* Draize test and the EYTEX™ method for 17 substances. No explanations were able to account for the lack of correlation since the vendor was able to duplicate the results in their laboratory.

# 5 CONCLUSIONS

This report has attempted to illustrate some of the alternatives to the Draize test in order to provide some background necessary for discussion and evaluation.

In spite of the wealth of research conducted to refine the Draize test, it is unlikely that the test will be modified to the point where all pain and stress to the animal is alleviated. In

addition, some tests, such as measurements of corneal thickness, require the use of specialized equipment, additional expense, and special skills (Walberg, 1983). Ethical considerations for the humane treatment of the test animals will always be an important criterion for the continuation of the Draize test. As suggested by Talsma *et al.* (1988), consideration should be given to the development of criteria for the disqualification of testing of any substance for eye irritancy. The Draize test should be viewed only as a last resort. As well, the Draize test results as reported in the scientific literature or in independent studies serve as a basis for evaluation for the *in vitro* tests. However, the Draize results may not accurately reflect the chemical's effects on human subjects. Because of this and other factors discussed, the lack of correlation between the *in vivo* and *in vitro* tests may not be reflective of a less predictable test for humans (van Erp and Weterings, 1990).

Although many *in vitro* alternatives have been developed, the evidence supporting their use as substitutes for the Draize test is insufficient. For example, validation of the *in vitro* tests has not progressed to the point where regulatory agencies and commercial industries are wholeheartedly supportive of their use (ECETOC, 1988). In order for these alternatives to be accepted, these tests need to be properly developed, proceed through a formal validation process, and the validation trials be independently reviewed (Balls and Clothier, 1991).

There are two major requirements for an alternative test to the Draize eye-irritancy test to be recognized and valid (Koch, 1989; Frazier *et al.*, 1987b):

1. demonstrated as reliable within and across different laboratories,
2. provide meaningful data with regard to chemical safety evaluation.

Recent research has attempted to validate these procedures as suitable alternatives to the Draize test (Bagley *et al.*, 1992; Blein *et al.*, 1991; Spielmann *et al.*, 1991; Kalweit *et al.*, 1990; Sterzel *et al.*, 1990; Boorman *et al.*, 1988). Boorman *et al.* (1988) examined 14 different *in vitro* eye irritation assessment procedures and found low correlation between the *in vitro* and Draize test results. They concluded that their study was only the beginning of a long scheme aimed at finding more humane methods of assessing eye irritation potential for chemical substances. Validation is a necessary but time-consuming and complicated process, (Balls and Clothier, 1991; Holden, 1988). The goals of the validation programs and the criteria for assessing the suitability of the alternative tests must be clearly defined before implementation of the program. Reference

chemicals must be selected for testing which cover a range of ocular irritancy (Bruner *et al.*, 1991).

Based on the research described above, several conclusions can be made:

a. The onus is on the researcher to demonstrate the need for the Draize test. It requires the researcher to justify the need for the procedure and to reduce the animal numbers as much as possible. Consideration must be given to previously published test results for chemicals with similar structures; demonstration via physical and chemical evaluations that a potential hazard exists; and negotiated regulatory requirements.

b. Most of the noninvasive Draize refinements are expensive. The equipment and technical expertise are not readily available. However, the fluorescein and exfoliative cytology evaluations could be implemented with minimal effort.

c. Replacement of the Draize test will not be satisfied by one test. Because of the complexity of the eye, *in vitro* tests are unable to duplicate exactly the responses of the eye to irritants. Therefore, alternatives to the Draize test will require a battery of tests, including cytotoxicity, cellular morphology, cellular metabolism, cellular physiology and repair (McCulley, 1985).

d. Development of the alternatives to the Draize test will require more knowledge about the specific cellular and molecular mechanisms that occur in chemically induced ocular injury (Bruner *et al.*, 1991).

e. The enucleated eye procedure may provide a means of screening chemicals for initial irritancy potential assuming that technical problems can be overcome.

f. Alternative tests will require validation before adoption as standard procedures.

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# APPENDIX A

## SCALE OF WEIGHTED SCORES FOR GRADING THE SEVERITY OF OCULAR LESIONS

(Draize *et al.*, 1944)

# APPENDIX A

## Scale of Weighted Scores for Grading the Severity of Ocular Lesions (Draize *et al.*, 1944)

1. CORNEA

A. Opacity - Degree of Density (area which is most dense is taken for reading)
- Scattered or diffuse area - details of iris clearly visible . . . . . 1
- Easily discernible translucent areas, details of iris slightly obscured . . . . . 2
- Opalescent areas, no details of iris visible, size of pupil barely discernible . . . . . 3
- Opaque, iris invisible . . . . . 4

B. Area of Cornea Involved
- One quarter (or less) but not zero . . . . . 1
- Greater than one quarter - less than one half . . . . . 2
- Greater than one half - less than three quarters . . . . . 3
- Greater than three quarters - up to whole area . . . . . 4

Score equals A x B x 5 Total maximum = 80

2. IRIS

A. Values
- Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive) . . . . . 1
- No reaction to light, hemorrhage; gross destruction (any one or all of these) . . . . . 2

Score equals A x 5 Total possible maximum = 10

3. CONJUNCTIVAE

A. Redness (refers to palpebral conjunctivae only)
- Vessels definitely injected above normal . . . . . 1
- More diffuse, deeper crimson red, individual vessels not easily discernible . . . . . 2
- Diffuse beefy red . . . . . 3

B. Chemosis
- Any swelling above normal (includes nictitating membrane) . . . . . 1
- Obvious swelling with partial eversion of the lids . . . . . 2
- Swelling with lids about half closed . . . . . 3
- Swelling with lids about half closed to completely closed . . . . . 4

C. Discharge

Any amount different from normal (does not include small amount observed in inner canthus of normal animals) . . . . . 1

Discharge with moistening of the lids and hairs just adjacent to the lids . . . . . 2

Discharge with moistening of the lids and considerable area around the eye . . . . . 3

Score (A + B + C) x 2 Total maximum = 20

The maximum total score is the sum of all scores obtained for the cornea, iris and conjunctivae.

## APPENDIX B

## SCALE FOR ESTIMATION OF THE SEVERITY OF CORNEAL LESIONS
(Friedenwald *et al.*, 1944)

# APPENDIX B

## Scale for Estimation of the Severity of Corneal Lesions
(Friedenwald *et al.*, 1944)

| Symptom | | Maximum Grade, Points |
|---|---|---|
| Corneal opacity | | |
| Intensity | | 8 |
| Area | | 4 |
| Less than one fourth of cornea | = 1 | |
| Less than one half of cornea | = 2 | |
| Less than three fourths of cornea | = 3 | |
| Over three fourths of cornea | = 4 | |
| Duration | | 4 |
| 1 to 3 days | = 1 | |
| 4 to 6 days | = 2 | |
| 7 to 13 days | = 3 | |
| 14 days and over | = 4 | |
| Corneal edema or bulge (seen with hand slit lamp and loupe) | | 4 |
| Corneal slough or ulceration | | 4 |
| Denuded epithelium | = 1 | |
| Moderate slough | = 2 | |
| Pronounced slough | = 3 | |
| Perforation | = 4 (100% lesion) | |
| Pannus (including density and length) | | 4 |
| Conjunctiva | | |
| Redness | | 2 |
| Edema | | 2 |
| Necrosis | | 2 |
| Discharge | | 2 |
| Iritis | | 4 |
| Small pupil and photophobia | = 1 | |
| Congestion of iris or positive aqueous ray | = 2 | |
| Exudative iritis | = 3 | |
| Panophthalmitis | = 4 | |
| | | 40 x 2.5 |
| | | 100% |